J. Clin. Chem. Clin. Biochem.
Vol. 19, 1981, pp. 291–294

Unconjugated 5α-Androstan-3α, 17β-diol and 5α-Androstane-3β, 17β-diol in Human Plasma as Measured by Radioimmunoassay without Chromatography

By *J. Tamm* and *U. Volkwein*

2. Medizinische Universitätsklinik, Hamburg

(Received August 18/December 4, 1980)

Summary: A radioimmunoassay (RIA) without chromatography for the determination of 5α-androstan-3α, 17β-diol and of 5α-androstane-3β, 17β-diol in human plasma by using highly specific antisera against the 15β-carboxyethylmercapto-bovine serum albumin conjugates is described. It could be shown that the 13% cross reaction of the 5α-androstane-3β, 17β-diol antiserum with pure androst-5-ene-3β, 17β-diol was negligibly low in plasma extracts. Sensivity, accuracy, precision, and linearity revealed results which comply with the requisites of a reliable RIA. The plasma levels obtained were as follows (mean ± SD): 5α-androstan-3α, 17β-diol: Normal males (N = 27) 0.86 ± 0.22 nmol/l; normal females (N = 10) 0.31 ± 0.07 nmol/l; hirsute females (N = 25) 0.60 ± 0.24 nmol/l; 5α-androstane-3β, 17β-diol: Normal males 1.47 ± 0.43 nmol/l; normal females 0.49 ± 0.10 nmol/l; hirsute females 0.91 ± 0.32 nmol/l.

Unkonjugiertes 5α-Androstan-3α, 17β-diol und 5α-Androstan-3β, 17β-diol im Plasma des Menschen, mit Radioimmunoassay ohne Chromatographie bestimmt

Zusammenfassung: Es wird ein Radioimmunoassay ohne Chromatographie zur Bestimmung von 5α-Androstan-3α, 17β-diol und 5α-Androstan-3β, 17β-diol im menschlichen Plasma beschrieben. Er basiert auf hoch-spezifischen Antisera gegen die 15β-Carboxyethylmercapto-Rinderserumalbumin-Konjugate dieser Steroide. Die 13%ige Kreuzreaktion des 5α-Androstan-3β, 17β-diol-Antiserums gegen reines Androst-5-en-3β, 17β-diol war extrem niedrig mit Plasma-Extrakten. Empfindlichkeit, Richtigkeit, Genauigkeit und Linearität entsprechen den Kriterien eines zuverlässigen RIA. Die gemessenen Plasma-Konzentrationen waren folgende ($\bar{x}$ ± s): 5α-Androstan-3α, 17β-diol: Normale Männer (N = 27) 0,86 ± 0,22 nmol/l; normale Frauen (N = 10) 0,31 ± 0,07 nmol/l; hirsute Frauen (N = 25) 0,60 ± 0,24 nmol/l; 5α-Androstan-3β, 17β-diol: Normale Männer 1,47 ± 0,43 nmol/l; normale Frauen 0,49 ± 0,10 nmol/l; hirsute Frauen 0,91 ± 0,32 nmol/l.

Introduction

Owing to the increasing interest in the metabolism of 17β-hydroxy-5α-androstan-3-one in androgenic target organs, the development of a simple and specific procedure for the estimation of the two important metabolites of 17β-hydroxy-5α-androstan-3-one, 5α-androstan-3α, 17β-diol and 5α-androstane-3β, 17β-diol, in plasma was attempted. The methods published so far (for references see discussion) need chromatographic steps for separating cross reacting steroids. This disadvantage was overcome by using specific antisera raised against the 15β-carboxyethylmercapto-bovine serum albumin conjugates of 5α-androstan-3α, 17β-diol and 5α-androstane-3β, 17β-diol (1). The methodological details are described in this paper.

Material and Methods

All reagents were analytical grade and used without further purification.
[1,2-^{3}H]5α-androstan-3α, 17β-diol (spec. activity 1.48 to 2.22 TBq/mmol) and [1,2-^{3}H]androst-5-ene-3β, 17β-diol (spec. activity 1.48 to 2.22 TBq were obtained from NEN (Dreieich, F.R.G.). [1,2-^{3}H]5α-androstane-3β, 17β-diol (spec. activity 1.48 to 2.22 TBq/mmol) was purchased from Amersham-Buchler (Braunschweig, F.R.G.). The purity of labelled steroids was checked every 6 weeks. Non-labelled steroids were purchased from Steraloids (Wilton, U.S.A.).

Antisera against the 15β-carboxyethylmercapto-bovine serum albumin conjugates of 5α-androstan-3α, 17β-diol and 5α-androstane-3β, 17β-diol were obtained by courtesy of Dr. Rao (San Antonio, U.S.A.).

A RIA was adapted for the estimation of 5α-androstan-3α, 17β-diol and 5α-androstane-3β, 17β-diol in plasma. The details of the method are as follows:

0340-076X/81/0019-0291$02.00

To 2 ml heparinized plasma, 10 µl of an ethanolic solution of [^{3}H]5α-androstan-3α,17β-diol (33.3 Bq) were added, followed by an extraction with diethyl ether (10 volumes per volume). The aqueous phase was frozen at – 18 °C and the ether siphoned off and evaporated under nitrogen. The residue was dissolved in 2.2 ml physiological saline and 150 µl were used for the recovery determination. Duplicate estimations of 3α- and 3β-diol were performed in 500 µl samples. To each 500 µl specimen, 500 µl of a physiological saline solution of [^{3}H]5α-androstan-3α,17β-diol or [^{3}H]5α-androstane-3β,17β-diol (333 Bq), respectively, were added together with 250 µl of the respective 5α-androstan-3α,17β-diol or 5α-androstane-3β,17β-diol antiserum dissolved in a solution of bovine serum albumin (1 g/l (working titer 1:8000). The period of incubation at 4 °C was 16 hours. Separation of free and bound steroid moieties was achieved by the addition of 200 µl of a charcoal/dextran solution (500 mg/100 ml charcoal; 50 mg/100 ml dextran; 100 mg/100 ml γ-globulin in physiological saline). The mixture was incubated for 20 minutes at 4 °C, and thereafter centrifuged for 6 minutes (2000 *g*). The supernatant was transferred into counting vials and to each sample 10 ml Insta-Gel® (Packard) were added.

Scintillation counting was accomplished by means of a Betaszint-5000 (Berthold, Wildbad, F.R.G.) on-line with a Diehl Alphatronic desk computer (tolerated error less than 8%).

Standard curves were prepared in triplicate for each series of determinations, using 0 to 1500 pg of the respective non-labelled steroid (fig. 1 and 2).

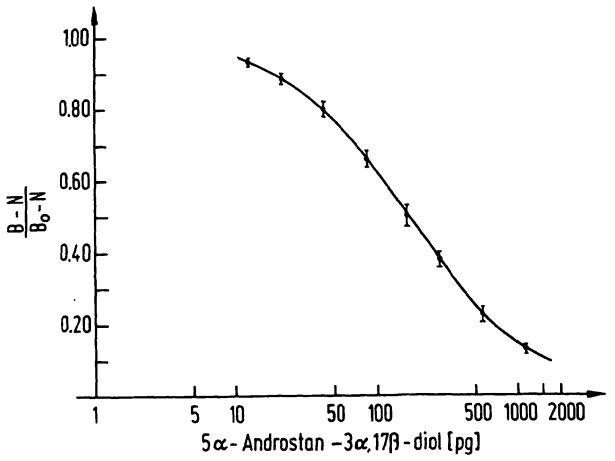

Fig. 1. Standard curve (mean ± SD) of 5α-androstan-3α,17β-diol obtained from 10 curves within 8 weeks.

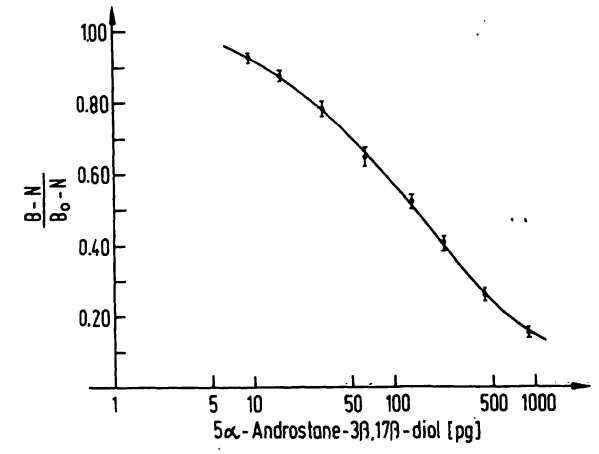

Fig. 2. Standard curve (mean ± SD) of 5α-androstane-3β,17β-diol obtained from 11 curves within 8 weeks.

Epoxidation of androst-5-ene-3β,17β-diol

The method reported by *Lisboa* (2) was used with slight modifications. Ethanolic solutions containing 1000 pg of cold 5α-androstane-3β,17β-diol and androst-5-ene-3β,17β-diol, respectively, were evaporated to dryness and 1 ml of *p*-nitroperbenzoic acid in diethyl ether added (10 g/l). The solution was left for 17 hours at room temperature and thereafter diluted with 5 ml diethyl ether and washed by sucessive addition of 2 ml of a sodium bicarbonate solution (100 g/l), 3 × 2 ml redistilled water and 1.5 ml of a saturated NaCl solution. The diethyl ether phase was evaporated to dryness and redissolved in physiological saline for 5α-androstane-3β,17β-diol RIA as described in the foregoing section. [^{3}H]5α-androstane-3β,17β-diol, [^{3}H]androst-5-ene-3β,17β-diol and mixtures of both steroids in ethanol (33.3 Bq) were brought to dryness and treated with *p*-nitroperbenzoic acid in the manner described above. The final residue was separated by TLC (cyclohexane/ethyl acetate; 40 ml + 60 ml) and scanned by means of a Beta-camera (Berthold, Wildbad, F.R.G.).

Paper chromatography

In order to separate 5α-androstane-3β,17β-diol and androst-5-ene-3β,17β-diol, the *Bush* A system (heptane/methanol/water; 50 ml + 40 ml + 10 ml) was used (running time 30 h at 27 °C; Schleicher and Schüll paper 2043b). The location of the steroids was achieved by labelled standards (333 Bq) placed on parallel strips and scanning with a Berthold paperscanner. The corresponding sections of the strips containing the plasma extracts were cut out and eluted with methanol. 5α-androstane-3β,17β-diol RIA was performed on the dried residues as outlined above.

Statistics

Statistical calculations were performed according to the *Student*'s t-test.

Results

Specificity

The extremely high specificity of the 5α-androstan-3α,17β-diol antiserum reported by *Rao* et al. (1) could be confirmed by control experiments in our laboratory. Moreover, the low cross-reaction with androsterone of 1.22% was found to be less than 1% in our experiments. The 5α-androstane-3β,17β-diol antiserum also proved to be remarkably specific. The data of cross-reactions with 5α-dihydrotestosterone (1.25%), testosterone (0.38%), oestradiol-17β (1.35%), and oestriol (0.11%) published by *Rao* et al. (1) turned out to be half as high on average. The list of tested compounds has been completed in that also the 17α-hydroxy-isomers of the androstane-diols (0.13%) and the 5α,6α epoxy derivative of 5α-androstane-3β,17β-diol (0%) were included. However, the cross-reaction with androst-5-ene-3β,17β-diol amounted to 13% in our experiments compared to 15.27% found by *Rao* et al (1). Therefore, efforts have been made to remove this interfering steroid. For this purpose the epoxidation by treatment with *p*-nitroperbenzoic acid (2) was chosen. Excellent results were obtained with pure [^{3}H]5α-androstane-3β,17β-diol and [^{3}H]androst-5-ene-3β,17β-diol, 99,5% of [^{3}H]androst-5-ene-3β,17β-diol being converted to [^{3}H]5α,6α-epoxy-androstane-3β,17β-diol, whereas the [^{3}H]5α-androstane-3β,17β-diol remained unaffected as could be shown by TLC. However, the plasma extracts contained unknown impurities following the epoxidation

Tab. 1. Mean concentration of 5α-androstane-3β,17β-diol extracted from a male plasma pool.
A = RIA without chromatography
B = RIA following separation of androst-5-ene-3β,17β-diol by paper chromatography.

	A	B
Number of samples	5	5
Mean (nmol/l) ± SD	1.09 ± 0.09	1.04 ± 0.11
Coefficient of variation (%)	8.3	10.4

reaction which made it impossible to get correct displacement curves. Therefore, experiments were carried out to compare the 5α-androstane-3β,17β-diol RIA with extracts from male plasma before and after separation of 5α-androstane-3β,17β-diol and androst-5-ene-3β, 17β-diol. The separation of the two 3β-hydroxy-steroids by paper chromatography in the System *Bush* A was excellent (running distance 9.3 and 11.8 cm, respectively). The results of the RIA performed with five respective plasma samples are demonstrated in table 1. It is apparent that after chromatography the concentration of 5α-androstane-3β,17β-diol was 96% of that determined "directly". This insignificant difference encouraged us to carry out the 5α-androstane-3β,17β-diol RIA also without preceding chromatography. The steroid concentration eluted from the androst-5-ene-3β,17β-diol spots and estimated with the 5α-androstane-3β,17β-diol antiserum amounted to 0.03 nmol/l.

Sensitivity

The sensitivity was estimated using the formula of *Abraham* (3):

$$S = \frac{2 \times SD}{R \times F} \times 100$$

were R is the percent recovery, F is the fraction of the recovered steroid used in the assay, and SD is the standard deviation of the mean of quadruplicate blank values. The blank values for 5α-androstan-3α,17β-diol RIA were $\bar{x}$ 2.31 ± 4.3 pg (N = 15) and the sensitivity 7.0 pg; the corresponding figures for the 5α-androstane-3β, 17β-diol RIA were $\bar{x}$ 0.73 ± 2.2 pg and 3.0 pg. Figures 1 and 2 show the mean standard curves (± SD) for 5α-androstan-3α,17β- and 5α-androstane-3β,17β-diol, obtained from 10 different curves within a period of 8 weeks.

Accuracy

33.3 Bq of [^{3}H]5α-androstan-3α,17β-diol were added to 37 samples of preextracted female plasma. The recovery was found to be $\bar{x}$ 92.1% ± 9.05 (SD) and the coefficient of variation (CV) calculated to be 9.82%. The corresponding data for [^{3}H]5α-androstane-3β,17β-diol using the same procedure were $\bar{x}$ 92.9% ± 8.95; CV 9.63%. To four preextracted plasma samples, different amounts of non-labelled 5α-androstan-3α,17β-diol and 5α-androstane-3β,17β-diol were added. The results are presented in table 2. In these experiments, the

Tab. 2. Mean values of recovered 5α-androstan-3α,17β-diol and 5α-androstane-3β,17β-diol from preextracted male plasma (N = 4).

5α-androstan-3α,17β-diol			5α-androstane-3β,17β-diol		
Added (pg)	Recovery (pg)	(%)	Added (pg)	Recovery (pg)	(%)
472.0	421.0	89.1	0	< 3	
50.9	41.0	80.6	0	< 3	
560.0	553.0	98.7	36.4	37.1	102.8
50.9	43.3	85.0	764.0	797.0	104.3
0	< 7		764.0	773.0	101.2
0	< 7		36.4	32.0	88.0

recovery of low and high concentrations revealed somewhat better results with the 5α-androstane-3β,17β-diol antiserum. The figures show clearly that no cross-reaction between 5α-androstan-3α, 17β-diol and 5α-androstane-3β, 17β-diol existed.

Precision

Calculation of the intra-assay variation of the 5α-androstan-3α,17β-diol RIA yielded the following results (mean ± SD): 0.18 ± 0.02 nmol/l; CV = 9.31% (N = 10) and 0.42 ± 0.02 nmol/l; CV = 5.54% (N = 7). The corresponding data for the 5α-androstane-3β,17β-diol RIA were as follows: 0.39 ± 0.03 nmol/l; CV = 8.59% (N = 10) and 1.08 ± 0.07 nmol/l; CV = 6.21% (N = 8). The inter-assay variation of the 5α-androstan-3α,17β-diol RIA came to 0.34 ± 0.03 nmol/l; CV = 8.15% (N = 8) and of the 5α-androstane-3β,17β-diol RIA to 0.56 ± 0.04 nmol/l; CV = 6.41% (N = 8).

Linearity

In seven different aliquots (20 to 150 µl) of a male plasma pool, 5α-androstan-3α,17β-diol and 5α-androstane-3β,17β-diol were estimated and revealed rectilinear curves (5α-androstan-3α,17β-diol: r = 0.99; 5α-androstane-3β,17β-diol: r = 1.01).

Plasma concentrations

The mean plasma levels obtained in normal adult male and female subjects, as well as in females with idiopathic hirsutism are compiled in table 3. The differences be-

Tab. 3. Plasma concentrations of 5α-androstan-3α,17β-diol and 5α-androstane-3β,17β-diol in normal male and female subjects and in females with idiopathic hirsutism.

	Age (range)	5α-androstan-3α, 17β-diol (nmol/l) mean ± SD	5α-androstane-3β, 17β-diol (nmol/l) mean ± SD
Normal males (N = 27)	17–50	0.86 ± 0.22	1.47 ± 0.43
Normal females (N = 10)	27–50	0.31 ± 0.07	0.49 ± 0.10
Idiopathic hirsutism (N = 25)	17–45	0.60 ± 0.24	0.91 ± 0.32

tween males and females, and between females and hirsutes were found to be highly significant: Males versus females: 5α-androstan-3α,17β-diol $p < 0.001$; 5α-androstane-3β,17β-diol $p < 0.001$. Females vs. hirsutes: 5α-androstan-3α,17β-diol $p < 0.01$; 5α-androstane-3β,17β-diol $p < 0.001$.

Discussion

5α-androstan-3α,17β-diol

The high specificity of the 5α-androstan-3α,17β-diol antiserum raised by *Rao* et al. (1) was confirmed. The methodological details presented fulfilled all criteria for a reliable RIA without chromatography in plasma extracts. Until now, no "direct" RIA for 5α-androstan-3α,17β-diol in plasma has been published. The results obtained in normal males and females correspond very well with those reported by *Habrioux* et al. (4). However, the data are higher compared than those published previously by others (5, 6, 7) and from our laboratory (8), using different types of chromatography and less specific antisera. The reason for these differences remains obscure.

5α-androstane-3β,17β-diol

No "direct" RIA has been described hitherto for 5α-androstane-3β,17β-diol in plasma. The specificity of the the 5α-androstane-3β,17β-diol antiserum obtained from *Rao* et al. (1) seemed to be reduced owing to cross-reaction of 13% with androst-5-ene-3β,17β-diol, which has been estimated in male plasma in rather high concentrations ranging from 2.28 to 5.47 nmol/l depending upon the specificity of the antiserum and the method of separation used (9, 10, 11, 12, 13). Unfortunately, the removal of androst-5-ene-3β,17β-diol by epoxidation (2) gave good results only with pure steroids; but introduced unknown factors which inhibited the displacement reaction with plasma extracts. It could be shown, however, that following separation of 5α-androstane-3β,17β-diol and androst-5-ene-3β,17β-diol by paper chromatography the difference between the figures before and after this procedure was only 4%. The 5α-androstane-3β,17β-diol concentration of the androst-5-ene-3β,17β-diol spots was 0.03 nmol/l. This would correspond to only 0.8% of unconjugated androst-5-ene-3β,17β-diol in male plasma. If the 5α-androstane-3β,17β-diol antiserum of *Rao* et al. (1) also exhibited a 13% cross-reaction with plasma androst-5-ene-3β,17β-diol, the results presented in this paper should be higher at least by 0.5 nmol/l in males and by 0.27 nmol/l in females. Obviously, the cross-reaction with plasma androst-5-ene-3β,17β-diol is negligibly low. Similar results were obtained in plasma drawn from spermatic vein (unpublished). The conclusion can be drawn that in some instances the cross-reaction of a steroid estimated with added pure steroid does not necessarily correspond with the cross-reaction of the same steroid extracted from plasma. This statement is confirmed by comparing the results described above with those reported in the literature which are markedly higher: *Hopkinson* et al. (14) found 2.08 and 0.97 nmol/l of plasma 5α-androstane-3β,17β-diol in males and females, respectively. The corresponding data reported by *Habrioux* et al. (4) were 2.79 and 1.76 nmol/l. Regarding sensitivity, accuracy, precision, and linearity the 5α-androstane-3β,17β-diol RIA met all requirements of a reliable method. Therefore, both antisera of *Rao* et al. (1) are suitable tools for a "direct" RIA of 5α-androstan-3α,17β-diol and 5α-androstane-3β,17β-diol in human plasma.

Acknowledgements

The experiments were supported by the Deutsche Forschungsgemeinschaft (Sonderforschungsbereich Endokrinologie, 34). Antisera against 5α-androstan-3α,17β-diol and 5α-androstane-3β,17β-diol were generously supplied by Prof. *Rao* (San Antonio, Texas). Thanks are extended to Dr. *Pirke* (München) who placed a specimen of his androst-5-ene-3β,17β-diol antiserum at our disposal for comparative experiments. The valuable advice of Prof. *Lisboa* is gratefully acknowledged.

References

1. Rao, P. N., Khan, A. H. & Moore, Ph. jr. (1977), Steroids *29*, 171–184.
2. Lisboa, B. P. (1969), Methods in Enzymology XV (Clayton, R. B. ed), Acad Press, New York 3–145.
3. Abraham, G. E. (1974), Acta Endocrinol. (Kbh) *75*, Suppl. *183*.
4. Habrioux, G., Desfosses, B., Condom, R., Faure, B. & Jayle, M. G. (1978), Steroids *32*, 61–71.
5. Kinouchi, T. & Horton, R. (1974), J. Clin. Endocrinol. Metab. *38*, 262–268.
6. Barberia, J., Pages, L. & Horton, R. (1976), Fert. Ster. *27*, 1101–1104.
7. Klemm, W., Liebich, H. M. & Gupta, D. (1976), J. Clin. Endocrinol. Metab. *42*, 514–519.
8. Laband, P., Tresguerres, J. A. F., Lisboa, B. P., Volkwein, U. & Tamm, J. (1978), Acta Endocrinol. (Kbh) *88*, 778–786.
9. Rosenfield, R. L. & Otto, P. (1972), J. Clin. Endocrinol. Metab. *35*, 818–822.
10. Loriaux, D. L. & Lipsett, M. B. (1972), Steroids *19*, 681–688.
11. Demisch, K., Magnet, W., Neubauer, M. & Schöffling, K. (1973), J. Clin. Endocrinol. Metab. *37*, 129–134.
12. Pirke, K. M. (1977), Steroids *30*, 53–60.
13. Forti, G., Calabrese, E., Gianotti, P., Borelli, D., Gonelli, P., Barbieri, U. & Serio, H. (1978), Horm. Res. *9*, 194–200.
14. Hopkinson, C. R. N., Park, B. K., Johnson, M. W., Sturm, G., Steinbach, K. & Hirschhäuser, C. (1977), J. Ster. Biochem. *8*, 1253–1257.

Prof. Dr. J. Tamm
Medizinische Universitäts-Klinik
Martinistraße 52
D-2000 Hamburg 20